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Conformational analysis of a modified ribotetranucleoside triphosphate: m^6_2A-U-m^6_2A-U studied in aqueous solution by nuclear magnetic resonance at 500 MHz[1]

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ABSTRACT The complete and unequivocal assignment of the 24 ribose proton signals of m^6_2A(1)-U(2)-m^6_2A(3)-U(4) by means of 500 MHz NMR spectroscopy at 17°C is given. This assignment is based on scrupulous decoupling experiments carried out at various temperatures. Analysis of the observed chemical shifts and coupling constants of the tetramer shows that the two fragments -m^6_2A(3)-U(4) comprising the 3'-end occur mainly in the classical right-handed stack conformation, whereas at the 5'-end the -U(2)- residue appears bulged out in favour of a less well-defined stacking interaction between the bases m^6_2A(1)- and -m^6_2A(3)-. Conformational populations about each of the torsional degrees of freedom along the backbone are discussed. A modernized version of pseudo-rotation analysis is used to delineate the conformational behaviour of the four ribose rings.

INTRODUCTION[2]

The rather unique vertical base-base stacking interaction exhibited by polynucleotides to form a single-helical array is understood to be one of the major driving forces in the formation of stable three-dimensional structures of RNA and DNA.

Proton NMR spectroscopy, carried out at sufficiently high resolution, is capable of yielding intimate geometrical details of backbone and sugar ring in each of the various subunits along the chain by means of a study of the proton-proton and proton-phosphorus coupling constants. Chemical shifts can yield additional data for the destack $\rightleftarrows$ stack equilibrium by monitoring individual protons, especially those protons that are most sensitive toward the overall change in conformation when going from the blend of unstacked microstates at high temperatures to one (or more) stacked state(s) at low temperature. Unfortunately, almost all the detailed NMR investigations that have been published up to date have been carried out on dinucleoside monophosphates (dimers), NpN. It should be realized that these compounds represent at the same time 3'- and 5'-terminal fragments of RNA and DNA, thus constitute a limiting case. Therefore, attempts at straightforward transfer of any conformational property of these dimers to longer fragments must be viewed

with caution. Indeed, optical studies[3] serve as a clear warning against gratuitous assumptions of additivity of properties at the trimer level in several specific cases.

The first complete proton NMR analysis of a trimer, A-A-A, was claimed[4] in 1976, but a recent reinvestigation[5] showed that the 1', 2' and 3' signals of the Ap- and -pAp- residues had been incorrectly assigned in the original work[4]. Our complete assignment of the 21 deoxyribose protons of a trimer[6], d(T-T-A), measured at 360 MHz, was published at the same time. Nowadays, trinucleoside diphosphates are certainly amenable to routine 360-400 MHz investigations, but detailed reports wherein chemical shifts and coupling constants for all sugar protons are tabulated still remain rather scarce: examples are d(A-A-A)[7], C-C-A[8] and A-C-C[8].

The advent of a new generation of superconducting NMR spectrometers operating at 500 MHz for proton spectroscopy holds great promise for the near future. In the present communication we wish to show that the increased dispersion allows for the first time the detailed assignment and study of all 24 ribose proton signals in a ribotetranucleoside triphosphate, abbreviated AUAU[2]. Both adenine bases in this compound are 6-N-dimethylated and therefore the strand is unable to form a Watson-Crick base-paired duplex structure. Thus, an in-depth study of its vertical base-base stacking properties is allowed without additional complications. Because assignment of the complex proton signal pattern, even at the trimer level[4], by incomplete decoupling experiments aided by visual inspection is not free from pitfalls we prefer to present the complete homonuclear decoupling scheme used to arrive at the unambiguous localization of each proton signal of the tetramer. Quantitative interpretation of the coupling constant data in terms of N and S[9,10] and C4'-C5' conformational populations is greatly improved by the use of a generalized Karplus Equation, recently developed in our Laboratory[11-13]. This new equation allows explicit correction for the electronegativity and orientation of substituents.

NOMENCLATURE

The new α-ζ notation recommended to IUPAC-IUB[14] for the conformational description of the polynucleotide backbone is used in this work. The torsion angles are labelled α-ζ in the direction of the chain, starting with α at the P-O5' bond, i.e.:

P-O5'-C5'-C4'-C3'-O3'-P
α β γ δ ε ζ

The nucleotide units, the protons in each unit, the corresponding coupling constants as well as the torsion angles are indexed between brackets as shown: R(1)-Y(2)-R(3)-Y(4).

MATERIALS AND METHODS

AUAU (fig. 1) was synthesized by an improved phosphotriester approach[15,16]. After purification on a DEAE-Sephadex-A25 (HCO_3^--form) column, the compound was treated with Dowex cation-exchange resin to yield the sodium salt. The sample was lyophilized three times and finally dissolved in 99.95% D_2O.

^{1}H-NMR spectra at 500 MHz of AUAU (0.015 M in D_2O + 0.005 M EDTA, pD 7.5, internal reference 0.002 M TMA)[2] were recorded in Fourier transform mode on a Bruker WM-500 spectrometer, using an internal D_2O lock, at probe temperatures of -3, 4, 11, 17, 27, 42, 57 and 72°C. The free induction decay signals (128 accumulations, quadrature detection) were averaged on a Bruker Aspect-2000 data system, using a spectral window of 3800 Hz with 32 K data points. Resolution enhancement was achieved by means of a Gaussian window technique according to Ernst[17]; resulting line-widths were about 0.8 Hz.

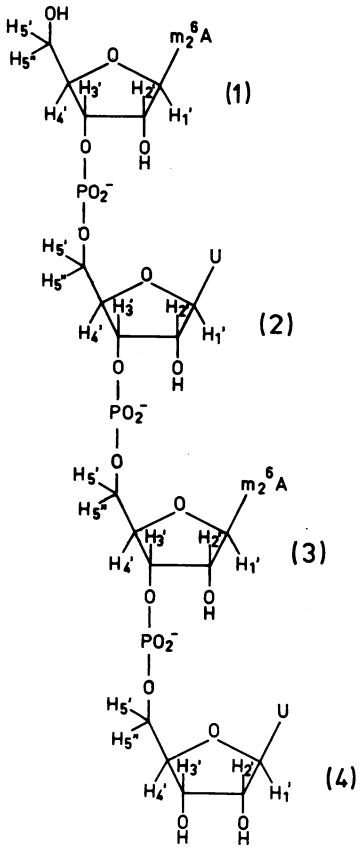
Extensive homonuclear decoupling experiments were carried out to assign the high field ribose signals, vide infra. Chemical shifts and coupling constant data at 17°C were obtained by simulation of the high field spectral region, using an expanded version of the computer program LAME.

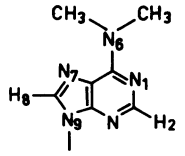
RESULTS AND DISCUSSION

Spectral assignment

The total ^{1}H-NMR spectrum of AUAU in D_2O consists of the signals of the base protons (singlets of the N6-methylgroups, H8 and H2 from A(1) and A(3), doublets of the H6 and H5 from U(2) and U(4)), and the more complex patterns of the 24 ribose protons H1'(1)-H5"(4) (fig. 1). Each ribose ring is characterized by a subset of six mutually coupled signals from H1'-H5". The first task in the assignment procedure is to settle which resonances belong to one and the same subset. Secondly, each subset must be assigned to one of the distinct fragments A(1) through U(4).

In an ideal case (all signals resolved) two decoupling experiments would be sufficient and necessary to link the six signals of a specific ribose residue (fig.2). When irradiations of respectively a distinct H2' and a H4' pattern cause the same H3' resonance to collapse, the concomitant changes of the corresponding H1', H5' and H5" signals complete the information needed to recognize one subset in the spectrum. The difficulties in pattern





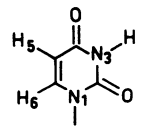


Figure 1. Structure of AUAU.

recognition encountered in practice due to overlapping resonances were overcome by carrying out decoupling experiments at various temperatures. When the temperature is changed in small steps (6-15°C), the signals of interest can

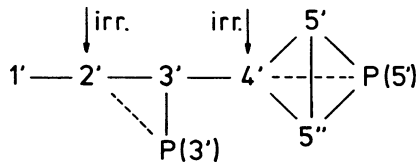


Figure 2. Strategy of homonuclear decoupling experiments. Dotted lines indicate long-range pathways.

be followed until they are sufficiently resolved to permit unambiguous conclusions at irradiation. Table 1 shows the results of the decoupling experiments A-M that gave clear changes a-m in the spectrum. It should be stressed that only positively interpretable observations are mentioned. Hence changes obscured by side bands near the irradiated frequency or observations that are ambiguous due to extensive overlap are not used in the assignment. The various subsets I through IV are assigned later to the fragments A(1)-U(4) (vide infra).

The signals of III are easily linked by the experiments D and M according to the assignment strategy of fig.2. The irradiations N, C, L and K identified the patterns that belong to II. Classification of the remaining resonances in two sets I and IV was less straightforward. The H3', H4', H5' and H5" signals of one group, say I, were recognized from experiments B and F. The irradiations A and E of the two H2' patterns not yet linked were decisive in an indirect way: in experiment A considerable sidebands prohibited the observation of a clear change in the H3' signal of I adjacent to the irradiated frequency. However, the latter was definitely not changed in experiment E, which did disturb (apart from a H1' doublet) a H3' quartet at much higher field. So the H1' and H2' patterns involved in experiment A belong to I. Finally, all the remaining signals constitute subset IV.

Pattern recognition of the H3' and H5', H5" resonances provided the main tool to associate the spectral groups I-IV to the molecular fragments A(1)--U(4). The H5', H5" signals form the AB part of either an ABM or an ABMX system, due to geminal coupling (JAB ≈ -12 Hz), vicinal coupling to H4' (J 2-5 Hz) and, only in case of 5'P substitution, vicinal coupling to phosphorus (JHX 2-6 Hz). In a similar way, a H3' signal shows up as part of an AMN or

Table 1. Decoupling experiments carried out at various temperatures: 17°C(A-G), 11°(K), 57°(L) and 72°C(M,N).
Capitals indicate irradiation at the proton signal indicated, corresponding lower case letters indicate clearly recognizable changes in signal pattern upon irradiation. Roman numerals refer to each subset of mutually coupled protons (see text).

	1'	2'	3'	4'	5'	5"
I	a	A	B	b,f	f	F
II	n	c,N	g,1,C	k,G,L	g	g,K
III	d	g,D	d,m,G	G,M	g,m	g
IV	e	E	e	-	-	-

AMNX pattern as a result of vicinal coupling to H2' (J 4-6 Hz), H4' (J 4-8 Hz) and, in case the pertinent fragment is 3'P substituted, to phosphorus (JHX 7-9 Hz). Therefore, the residues A(1) and U(4) are easily recognized in the spectrum as belonging to group I and IV, respectively. Both subsets are unique, each lacking a specific vicinal coupling to phosphorus present in the other three: the H5', H5" pattern is simplest in I, the H3' signal in IV (fig.4).

The remaining fragments U(2) and A(3) are both 3'P and 5'P substituted. Therefore, no distinction can be made between the residual subsets II and III on grounds of pattern recognition. Instead, the shifts of the H1' signals are considered. At increasing temperature the fast equilibrium between stacked and unstacked conformers will be shifted in favor of the unstacked molecules. This justifies comparison of AUAU with the constituent monomers. In this case the relevant compounds are mononucleoside 3',5'-diphosphates, measured at pD 5. The signal of the H1' in HOpUpOH is found at 0.1 ppm higher field than in HOpApOH (unmethylated)[5]. When the temperature is raised from 17°C the doublets of the two pertinent H1' protons in AUAU approach each other, cross at 42°C and are separated by 0.1 ppm at 72°C. Distinction remained possible due to the different magnitude of the coupling constants J1'2'. We feel safe to conclude that at 72°C the H1' signal at the highest field (group II) belongs to fragment U(2). Consequently, subset III corresponds to A(3), and this completes the unequivocal assignment of the 24 ribose proton signals of AUAU.

Experiment G serves as an independent check on a large part of the assignment based on the other experiments reported in Table 1. From fig.3 it can be seen that neither the H3' quartet (0.98 ppm) of the free 3'-OH fragments nor the H5' pattern (0.81 ppm) of the free 5'-OH residue has changed. So the irradiated H4' signals must belong to U(2) and A(3). Therefore, the only unaffected ABMX system (1.15 and 0.88 ppm) should be assigned to H5' and H5" of U(4). In addition, the clear reduction of the two other ABMX systems into ABX patterns permitted the proper determination of the coupling constants J5'P and J5"P in the fragments U(2) and A(3).

The assignment of the protons attached to the bases is more complicated. The adenine base protons H2 and H8 show singlets. The H2 signals are distinguished from the H8 resonances since H2 is non-exchangeable in D_2O with heating and also has a longer relaxation time T1. The H2 signal at the lowest field is tentatively assigned to fragment A(3). The H8 of A(3) appears at the lowest field due to the deshielding effect of the 5'-phosphate group. The four N6-methyl groups occur together in one broad resonance around 0 ppm. The

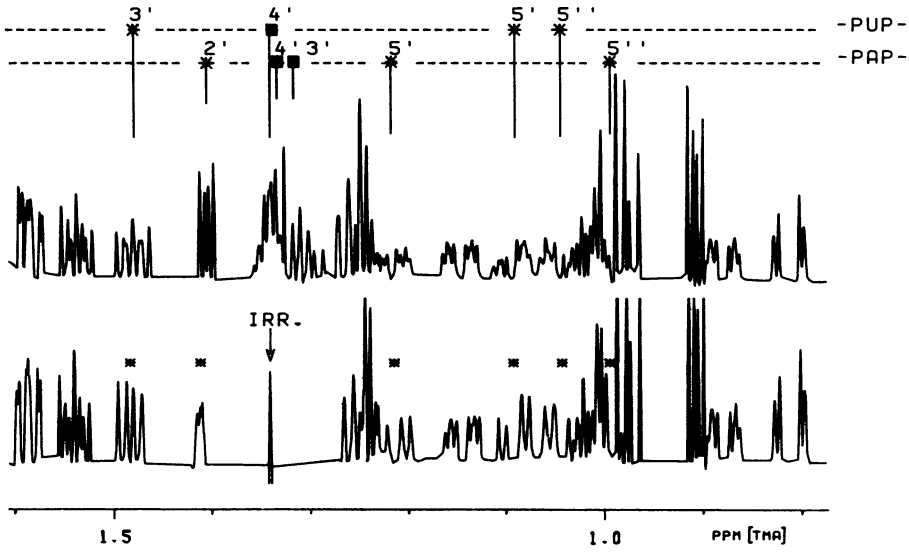


Figure 3. Part of the ribose region of the resolution-enhanced 500 MHz NMR spectrum of AUAU at 17°C. The spectra shown were digitized by hand for illustration purposes. Upper spectrum: nondecoupled. Lower spectrum: decoupled with arrow indicating the coupling frequency and asterisks denoting the decoupled signals. See also Table 1.

uracil residues of U(2) and U(4) each give rise to a doublet at about 4.3 and 4.7 ppm (H6) and 2.2 and 2.6 ppm (H5), with coupling constants J56 of 8.1 Hz. Irradiation of the H6 signal at 4.35 ppm caused the H5 doublet at 2.20 ppm to collapse into a singlet. The latter resonance at the highest field is assigned to H5 of U(4) on conformational grounds (vide infra).

The computer simulation of the spectrum of AUAU at 290 K is in excellent agreement with the experiment (fig.4). This confirms the assignment of the ribose ring proton signals. It is noted that coupling constants extracted from signals of various mutually coupled protons proved consistent. All chemical shift and coupling constant data are given in Tables 2 and 3.

Conformational Analysis

The exact conformation of each furanoid ring is determined by two parameters: the phase angle of pseudorotation P and the degree of pucker, Ψ_m[9,10,18]. Two conformation ranges are considered, type N ($P = 0^\circ \pm 90^\circ$, roughly C3'-endo) and type S ($P = 180^\circ \pm 90^\circ$, roughly C2'-endo). It is assumed that in solution two specific conformers N and S exist in fast equilibrium. By means of a modified Karplus equation[13] observed values for J1'2', J2'3'

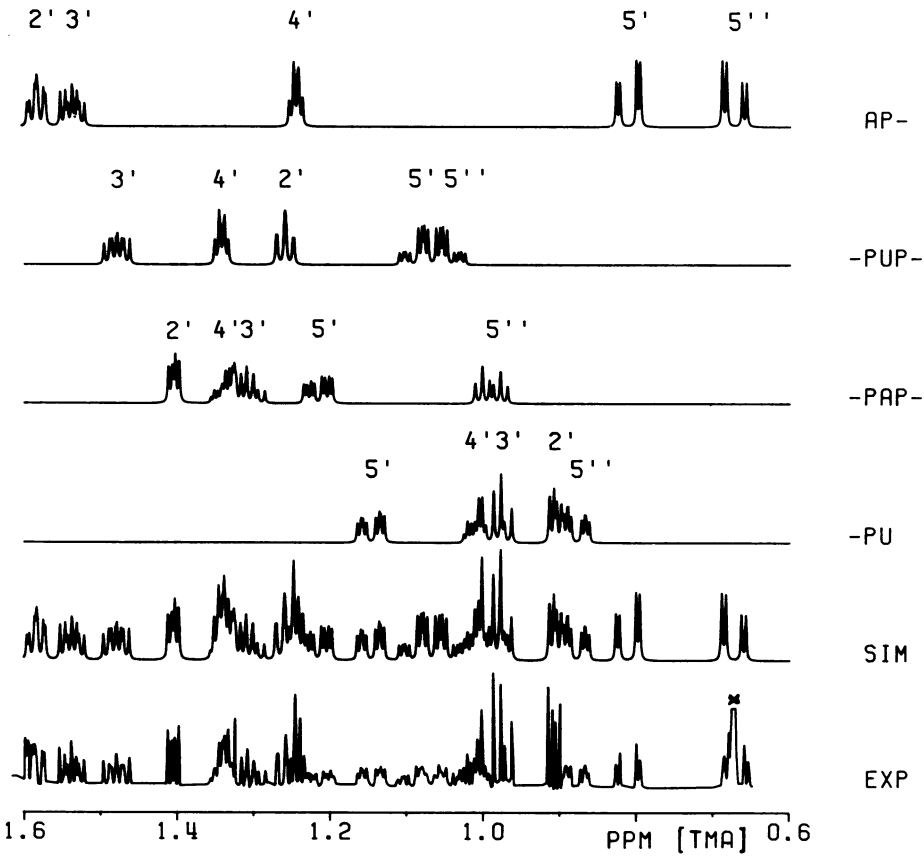


Figure 4. High-field part of the ribose region of the resolution-enhanced 500 MHz NMR spectrum of AUAU at 17°C. The experimental spectrum (denoted Exp) was digitized by hand for illustration purposes. The four upper spectra are computer simulations of the signals for each fragment as indicated in the margin. The definite assignment of the signals is also indicated. The spectrum denoted SIM displays the complete computer simulation. Asterisk in spectrum Exp indicates signal from added EDTA.

and J3'4' give experimental access to the pseudorotation parameters P(N), Ψm(N), P(S), Ψm(S) and the molefractions of N conformer X(N)[7,12]. However, when only data at a single temperature are available, the system is underdetermined: for each ribose ring five unknowns have to be solved from three observables. For the time being it is therefore assumed that the four ribose rings in AUAU adopt exactly the same N and S geometries in equilibrium. Only the relative amount of N conformer will be different in the various residues.

Table 2. Proton chemical shifts (δ in ppm) of the tetramer[2] AUAU at 17°C in D_2O. Concentration 0.015 M, pD 7.5. Shifts are given relative to the methyl peak of Me_4NCl. Estimated accuracy 0.002 ppm.

fragment	A(1)	U(2)	A(3)	U(4)
proton				
1'	2.708	2.711	2.622	2.414
2'	1.585	1.260	1.405	0.907
3'	1.538	1.480	1.308	0.976
4'	1.245	1.343	1.341	1.010
5'	0.810	1.089	1.216	1.147
5"	0.674	1.047	0.991	0.879
8 (6)	4.865	4.627	4.909	4.346
2 (5)	4.726	2.602	4.762	2.196

Furthermore, Ψm(N) and Ψm(S) are constrained to respectively 39° and 35°, values calculated from extensive analysis of more than one hundred coupling constants observed in AU and Am[19a]. The least squares computer program PSEUROT[19b] was used to calculate P(N), P(S) and for each ribose ring the mole fraction of N conformer (Table 4).

Table 3. Coupling constants (Hz) of AUAU at 17°C in D_2O. Estimated accuracy ± 0.1 Hz except where indicated by an asterisk (± 0.3 Hz).

fragment	A(1)	U(2)	A(3)	U(4)
coupling				
1'2'	5.8	5.8	2.5	2.9
2'3'	5.0	5.2	4.7	4.9
3'4'	3.4	3.7	7.3	7.1
4'5'	2.5	2.6	2.4	2.5
4'5"	2.8	2.7	3.8*	2.3
5'5"	-13.0	-11.8	-11.8	-11.8
2'P	1.3	0.8	- -	- -
3'P	7.6	7.9	8.5	- -
4'P	- -	2.5*	1.7*	2.5
5'P	- -	4.0	4.8	2.9
5"P	- -	4.6	4.9	3.5

Table 4. Population distributions (%) for the ribose rings and for the backbone angles β and γ in AUAU at 17°C.

	fragment	A(1)	U(2)	A(3)	U(4)
ribose[a]	N	27	30	79	74
β[b]	t	-	80	75	95
	g^+	84	84	74	89
γ[c]	t	14	12	25	8
	g^-	2	4	1	3

a) Calculated from J1'2', J2'3' and J3'4' by means of least-squares program PSEUROT. Ψ_N and Ψ_S were constrained at values of 35° and 39° respectively; X_N, P_N (17°) and P_S (161°) were refined[12,20].
b) Calculated from $\Sigma' = J5'P + J5''P$, see text.
c) Calculated from J4'5' and J4'5" according to Haasnoot et al[11]: $\gamma^+ = 51°$, $\gamma^t = 180°$, $\gamma^- = -70°$.

In the following discussion, AUAU is considered to be built up from two fragments AU linked by a phosphate group. It is of interest to compare the fragments A(1)U(2) and A(3)U(4) with the dinucleoside monophosphate AU. The latter compound has been the subjet of extensive CD and NMR studies[19a,20,21]. It appeared that at low temperatures in solution AU will adopt largely a classical right-handed stacked form (70% at 14°C). In this stacked form both ribose rings have a N type conformation[9,10,20,21]. From Table 4 it can be seen that in AUAU the A(3)U(4) fragment displays relative amounts of N conformer (79%, 74%) that agree well with those found in the dimer AU at 14°C, namely 80% N for both ribose rings[19a]. In contrast, the A(1)U(2) moiety displays a surprisingly low N content (29%, 30% respectively). This suggests that in the tetramer the -ApU3'OH fragment behaves quite analogous to the dimer ApU3'OH. On the other hand the behaviour of the 5'OHApU moiety deviates strongly from expectations. The striking difference in conformational properties between the two AU fragments is not only reflected by the N/S ribose populations but is also prominent in the chemical shifts. In the following discussion shifts from U(2) and U(4) serve as a probe to monitor stacking properties of these uridine residues with the nearest neighbour A(1) and A(3) residues. Use is made of differential shieldings by subtraction from data of relevant mononucleotides measured at (or interpolated to) 17°C. Positive values indicate shielding relative to the monomer.

In Table 5 the differential shieldings of the 3'-OH terminal uridine in AUAU and AU are shown. Shifting the destack $\rightleftarrows$ stack equilibrium of AU to the

Table 5. Differential shieldings (10^{-2} ppm) at 17°C for 3'-OH terminal uridine fragments in AUAU and AU, relative to iprU. Positive values indicate increased shielding.

proton	1'	2'	3'	4'	5'	5"	5	6
fragment								
AUAU	38	26	18	8	-18	0	56	41
AU	40	23	15	6	-22	-2	55	35
Δ	-2	3	3	2	4	2	1	6

right involves considerable shielding in the -pU moiety of H5, H6, H1', H2', H3' and deshielding of H5'[10]. The differential shieldings of AUAU are all very similar to those of AU. This drives us to the conclusion that neither the population distributions nor the geometries of the most predominant conformers of -ApU3'OH are essentially changed upon incorporation of this fragment as 3'-terminal end in longer RNA-fragments.

In case of A(1)-U(2) and U(2)-A(3) stacking in AUAU, the protons H6, H5 and H1' of U(2) should be shielded by the aromatic bases of both A(1) and A(3). Differential shieldings for the relevant uridine residues in AUAU, AU and UA are listed in Table 6. Surprisingly, the shieldings observed for the tetramer are 0.4 ppm (H6), 0.6 ppm (H5) and 0.7 ppm (H1') smaller than the sum of the shieldings predicted from the dimers. It is therefore concluded that residue U(2) is far less involved in stacking with any adenosine in AUAU than is expected from the constituent compounds AU and UA. The 5'-HOApU-fragment was found (vide supra) to have a surprisingly low content of N ribose ring conformers. Apparently there is no need for the ribose rings in this fragment to adopt an N conformation as is usual in a classical right-handed stack. This is in agreement with the remarkably low differential shieldings

Table 6. Differential shieldings (10^{-2} ppm) at 17°C for U(2) in AUAU compared with AU and UA. Reference monomers are Um and iprU[2].

fragment	AU	UA	AUAU	Δ
proton				
1'	40	39	7	-72
5	55	16	16	-55
6	35	17	13	-39

found for H6, H5 and H1' of U(2). It should be noted that reversal of the assignment of the two H5 signals would lead to inconsistencies for H5 in the overall picture of differential shieldings found in U(2) and U(4).

Table 7 shows differential shieldings of H8, H2 and H1' of the adenosine residues of AUAU, AU and UA. From the differential shieldings of AU and UA it can be seen that stacking of adenosine with a nearest neighbour uridine fragment does not lead to large shieldings of H8, H2 and H1' of the former residue. On the contrary, in all cases a small deshielding is observed (except for H1' (A) in AU). This emphasizes the significance of the observed differential shieldings of all H8 and H2 protons and H1' of A(3) in AUAU. It should be noted that the two H2 protons (or H8 protons) of AUAU remain both significantly shielded upon reversal of the assignment of the two H2 signals (or H8 signals) to A(1) and A(3). The observed extra shielding in the tetramer with respect to the dimers is explained by a next-nearest neighbour interaction of the two adenine bases in AUAU.

Recently Lee and Tinoco[5] investigated thirteen trinucleoside diphosphates by ^{1}H-NMR spectroscopy. Differential shieldings of base protons and H1' protons were compared with values predicted from constituent dimers and monomers. For the unmethylated compounds ACG, AUG and GUG deviations of 0.1-0.3 ppm were found. The central pyrimidine residue was less shielded and the two purine fragments were more shielded than expected. The authors proposed the existence of a conformation in which the interior base is "bulged out" and the two terminal bases stack on each other. It was stated that this conformation should be most pronounced for purine-pyrimidine-purine sequences, especially if the middle base is uracil.

From our investigation of AUAU it appears that the involvement of U(2) in stacking with nearest neighbours is strikingly small. In addition, a next--nearest neighbour interaction of the adenine bases can explain the observed

Table 7. Differential shieldings (10^{-2} ppm) at 17°C for A(1) and A(3) in AUAU compared with AU and UA. Reference monomers are Am and mA[2].

fragment	AUAU	AU	Δ	AUAU	AU+UA	Δ
proton						
1'	15	6	9	24	2	22
2	14	-1	15	9	-1	10
8	15	-7	22	19	-7	26

excess shieldings in A(1) and A(3). The deviation of the differential shieldings in fragment A(1)U(2)A(3) is far more pronounced than it is in ACG, AUG and GUG[5]. A considerable amount of conformers in which U(2) is "bulged out" and A(1) and A(3) have mutual stacking interaction must be present. It should be stressed that the stacking properties of fragment A(3)U(4) are nearly identical to those of the dimer AU (70% stack at 17°C). It is suggested that in longer chains the occurrence of bulging out exerts little or no influence on the conformational behaviour in the direction of the 3'-end. In solution at 17°C AUAU appears to display a considerable amount of conformers in which bulging out and regular right-handed stacking occur simultaneously (fig.5).

In the literature no details are known about specific structures of the backbone of a free oligonucleotide in which a bulged base appears. Only first -order values of J1'2' were obtained thus far[5].

In a ^{1}H-NMR study of ethidium complexes of selfcomplementary CUG (and GUG with CC) the existence of double helices with a bulged out uridine was proposed on grounds of chemical shift considerations[22]. No coupling constant data were published. However, it was stated that no large changes relative to the free trinucleoside diphosphates were observed in coupling constants that monitor sugar puckering and exocyclic linkages C4' - C5', C5' - O5' and C3' - O3'.

It is now for the first time that accurate coupling constants are used to obtain quantitative information about the backbone of an oligonucleotide in which both regular stacking and bulging out occur. Table 4 displays the com-

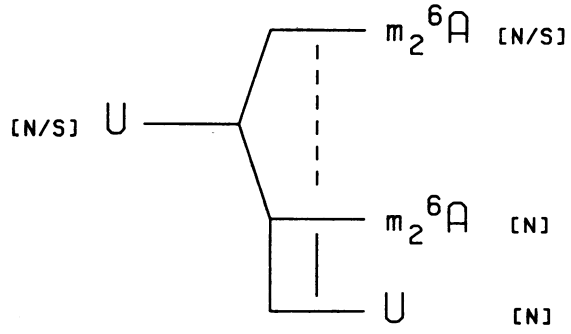


Figure 5. Highly schematic representation and ribose conformational properties of favoured nearest neighbour and next-nearest neighbour stacking interactions in AUAU.

position of the conformational equilibria of the ribose rings and the rotamer distributions around the C4' - C5' and C5' - O5' bonds.

Composition of the Ribose Conformational Equilibrium

The calculated N-conformer populations (vide supra) are remarkably different for the four fragments of AUAU. The outspoken preference for the N-type conformation in A(3) and U(4) is in accordance with the presence of at least 60% right-handed stack in the -ApU3'OH moiety at 17°C. In the fragments A(1) and U(2) a slight bias towards the S-type can be seen. The latter fragment is involved to a very minor degree in stacking interactions. Residue A(1) on the contrary stacks part of the time with its next nearest neighbour A(3). It is noteworthy that the amount of S-type ribose is the same in A(1) as in the monomer Am (75%). It is concluded, therefore, that residue A(1) retains the full freedom that it has in the corresponding monomer Am, i.e. it has no specific preference for N- or S-type ribose rings in those conformers in which both purines stack on each other.

Conformation around C4' - C5' (γ)

The population distribution of the three classical rotamers (γ^+, γ^-, γ^t) around the C4' - C5' bond can easily be calculated from the observed coupling constants J4'5' and J4'5". Limiting coupling constants for the individual rotamers are calculated according to Haasnoot et al.[11]. Attention is focused on the internucleotide C4' - C5' bonds. The γ^+ conformer predominates, whereas the amount of γ^- conformer is negligible in all fragments. The highest percentage γ^+ is found in U(4) because normal stacking requires γ^+ in this fragment. In U(2) and especially in A(3) the percentage γ^+ is considerably lower and comparable to the percentages calculated for the monomers mA and iprU (75±5%). We conclude that the existence of special conformers in the A(1)U(2)A(3) fragment is not reflected in higher conformational purity around any C4' - C5' bond.

Conformation around C5' - O5' (β)

The fractional population Pt of the preferred trans rotamer β^t around the C5' - O5' bond is calculated[23] from the formula Pt = (25-Σ')/20.8, where Σ' = J5'P + J5"P. The highest conformational purity is found between A(3) and U(4) due to the good stacking properties of fragment A(3)U(4). In contrast, the conformational freedom of β(2) and β(3) is similar to that in the monomers mA and iprU (75±5% β^t). It is concluded that the A(1)U(2)A(3) fragment has quite similar freedom around C5' - O5' bonds as found for the monomers.

Long-range coupling ^{4}JHP between ^{31}P and H4' is known to display a maximum value in a W-path configuration. In our case this means that ^{4}JPH4' will be

largest when γ^+ and β^t occur simultaneously in a given fragment. Two possibilities can be imagined for this situation to occur: (a) maximum cooperativity, PWmax: (b) no cooperativity, PWmin. Now, PWmax follows from either $P_{\gamma}+$ or $P_{\beta}t$, whichever population is the lowest, PWmin follows from the product $P_{\gamma}+.P_{\beta}t$. Analysis of the data (Table 8) shows a reasonable agreement with the predicted trend of ^{4}JHP with both PWmin and PWmax. It appears hazardous to draw conclusions concerning the question as which of the two models is the better one, but in any case the population PW lies between fairly narrow limits.

Conformation around C3' - O3' (ε)

From numerous X-ray diffraction studies it appears that the 3'-phosphate group in ribonucleotides definitely prefers two relatively narrow ranges of non-classical torsion angles: $\varepsilon \approx 220^{\circ}$ (t) and $\varepsilon \approx 260^{\circ}$ (g^-) with corresponding proton-phosphorus torsion angles of about -20° and $+20^{\circ}$, respectively. The g^+ rotamer has never been observed in the solid state and may therefore be excluded from consideration[24,25]. At first sight no distinction is possible between t and g^- on grounds of observed values for J3'P and a simple Karplus equation. This is due to the symmetrical position of H3' relative to phosphorus in both conformers. However, in AU a linear correlation of J3'P with the percentage stacking is found[19]. For the fully stacked form, in which ε must be t[24], a value of 9.7 Hz is calculated. For 0% stack J3'P extrapolates to 7.6 Hz. This value is indeed observed for the monomer Am at 17°C. Different absolute values of the proton-phosphorus torsion angle in the g^- and t conformer can account for this. In addition, a difference in ε^t between stacked and unstacked conformers may exist as observed in d(A-A) and d(A-A-A)[7]. Therefore, the pertinent coupling constants in AUAU can only be discussed in a qualitative way.

The values of J3'P (Table 3) indicate that the conformational behaviour of AUAU around ε(1) is similar to that of Am. Around ε(3) the rotamer distribu-

Table 8. Calculated populations of W-path ($\beta^t\gamma^+$) conformers in AUAU at 17°C and corresponding observed ^{4}J4'P values.

fragment	PWmin %	PWmax %	^{4}J4'P Hz
AUAU	66	79	2.5
AUAU	56	74	1.7
AUAU	79	89	2.5

tion is biased towards the situation in the stacked form of AU, as expected. The value of J3'P monitoring ε(2) is intermediate. In conformers with g^- around C3'- O3' and S ribose puckering H2' lies in an almost planar W configuration with the phosphorus atom at the O3' position. This configuration is detected for the residues A(1) and U(2) by long-range H2' - ^{31}P coupling constants of 1.3 Hz and 0.8 Hz, respectively. It is concluded that AUAU is comparable to monomers concerning the rotamer distribution around ε(1) and ε(2), and displays a high degree of freedom. Around ε(3) there is a bias towards the t conformer comparable to that observed in the dimer AU.

Conformation around O3' - P (ζ) and P - O5' (α)

Unfortunately, the torsion angles ζ and α cannot be monitored by means of coupling constants. However, the conformational freedom found for practically all the backbone angles in A(1)-U(2) and U(2)-A(3) presumably carries over to the phosphodiester linkages in these fragments as well, i.e. it seems probable that ζ(1), α(2) and ζ(2), α(3) are not confined to adopt a specific arrangement. This is of course not true for the stacked fragment A(3)pU(4), where one may expect a preference for the $\zeta^-\alpha^-$ conformer, i.e. a classical right-handed stack.

CONCLUSIONS

At the outset of this investigation several facts were known to us: (a) the 6-N-dimethylated compound AU displays excellent stacking properties, quite comparable to those of the fully 6-N-methylated AA dimer[19-21]; (b) the 6-N-dimethylated dimer UA supposedly cannot easily reach a state of perfect base-base overlap[27] and, indeed, the CD and NMR results[26] show that, at low temperatures, the destack $\rightleftarrows$ stack equilibrium is more shifted to the left (20^o lower Tm) in UA compared to AU; (c) the corresponding trimer UAU[26] displays enhanced stacking properties in the UA- part, whereas the -AU part behaves similar to the corresponding dimer. Extrapolation of these observations led to the expectation that the tetramer AUAU would consist of two well-stacked AU regions, perhaps separated by a weaker-stacked UA hinge. The present results do not conform to expectations: the 3'-terminal bases indeed retain to a large measure the stacking properties of the dimer (> 60% stack *vs* 70% stack respectively at 17^oC) but in sharp contrast the 5' AU- as well as the middle -UA- moieties show hardly any regular stack. From the coupling constants and differential shieldings one can estimate that the percentage of regular stack cannot exceed about 10%. Furthermore, the chemical shift *vs* temperature profiles (not shown) definitely indicate a non two-state behav-

iour of the chemical shifts in these residues, especially of the protons belonging to the -U(2)- unit.

One is now for the first time faced with the question why and how an energetically favourable stacking geometry in AU (ΔH = -6.5 kcal $mole^{-1}$, ΔS = -20.9 cal $mole^{-1}$ K^{-1})[21] can be abandoned by the oligonucleotide in favour of a bulging out of the central base in a purine-pyrimidine-purine sequence. One most pertinent fact has come to light in the present work: the complete conformational freedom displayed by the ribose rings (except A(3) ribose) and the backbone in the AUAU underlined part of the sequence. Because conformational freedom implies a significant gain in entropy of mixing it is hardly speculative to propose that the (1)...(3) stacking of the purine bases, concomitant with the gain in the entropy factor, suffices to offset the natural tendency for AU stacking. An exact calculation of the entropy of mixing is precluded for the time being because the true conformational populations along the P-O ester bonds ζ and α cannot be extracted from the data at hand, furthermore, the number of forbidden conformational combinations is not known. An upper limit for the entropic gain can easily be calculated from the backbone populations given in the present communication, with the additional assumption of roughly equal distribution of g^+, g^- and t along the phosphor diester bonds: $T\Delta S_{300} \sim 6$ kcal.$mole^{-1}$. Even a much smaller entropic gain would be sufficient to offset the loss of AU and UA stacking interactions in favour of a bulged out pyrimidine base with concomitant formation of a purine-purine (1)...(3) stack and restauration of conformational freedom along the backbone of two residues. Seen in this light, the bulged out situation does not represent a single well-defined conformation but rather an assembly of forms each of which is characterized by a purine-purine stacking interaction.

It is of interest to note that bulging out can be seen as a local disturbance of the regular stack. From the present work it appears that the bulge hardly interferes with normal stacking at the 3'-end.

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2. Abbreviations used:
CD, circular dichroism; NMR, nuclear magnetic resonance; TMA, tetramethylammonium chloride; Am, 6-N-(dimethyl)adenylyl-3'-O-methyl phosphate; Um, uridylyl-3'-O-methyl phosphate; iprU, uridylyl-5'-O-isopropyl phosphate; mA, 6-N-(dimethyladenylyl-5'-O-methyl phosphate; AU, 6-N-(dimethyl)adenylyl-(3'-5')-uridine; UA, uridylyl-(3'-5')-6-N-(dimethyl)adenosine; AUAU, 6-N-(dimethyl)adenylyl-(3'-5')-uridylyl-(3'-5')-6-N-(dimethyl)adenylyl--(3'-5')-uridine.
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